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## PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

TANAKA, Akiko; JESSIP, John; and BRADLEY, William Guy

Application No.: 09/964,240

Art Unit: 1654

Filed: September 26, 2001

Examiner: TATE, C.

For: PINE CONE EXTRACTS AND USES THEREOF

Conf. No.: 1854

Attorney Docket: 3974.002

DECLARATION UNDER 37 C.F.R. §1.131

Mail Stop Non-Fee Amendment  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

I, Akiko TANAKA, of 10900 Roosevelt Blvd., St. Petersburg, Florida 33716-2308, declare and state as follows:

1. I am a co-inventor of the invention disclosed and claimed in the above-identified application for U.S. Patent, am familiar with the contents of the Office Action mailed in regard to said application on March 24, 2003, and submit this Declaration for the purpose of establishing a date of invention prior to the date of publication of the reference Xin (CN 1279107), which is cited in said Office Action.

2. I am a research virologist possessing a Ph.D. in virology, and was co-founder in 1981 of the Tampa Bay Research Institute in St. Petersburg, Florida, where I am currently a Senior Member and Principal Investigator, and where I have been, since 1995,

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its President.

3. Prior to June 1, 1999, as the result of a collaboration between Akiko TANAKA, William Guy BRADLEY, and John JESSIP, (herein collectively "we"), I contributed to conception of the subject invention as disclosed and claimed in the above-captioned application for U.S. Patent.

4. On June 1, 1999, and in part as a result of the collaboration described in paragraph 4, a proposal for research funding naming William Guy BRADLEY, Ph.D., as Principal Investigator, and titled "Dissecting the Legendary Anti-Tumor Activity of a Pine Complex: Activating the Anti-Tumor Potential of Macrophage and Dendritic Cells," was submitted to the U.S. Army Medical Research and Materiel Command, and that a copy of this proposal and accompanying letter of submission is appended as Appendix A.

5. Receipt of the proposal of Appendix A was acknowledged by mail in the form of a postcard, a copy of which is appended as Appendix B, which postcard was mailed by the office of Dr. Kenneth A. Bertram, Lt. Col., U.S. Army Medical Corps, to Dr. William G. Bradley, assigning identification number BC990863 to the proposal.

6. The proposal of Appendix A sets forth, as described below, the essence of the invention of the above-captioned application for U.S. patent.

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7. I was aware from prior studies that activation of dendritic cells in vitro in the presence of tumor antigen and specific cytokines can induce tumor-specific cytotoxicity when the dendritic cells thus treated are used as a tumor vaccine. See Appendix A at page 8, lines 4-5.

8. In the proposal of Appendix A, we proposed the hypothesis that the host-mediated anti-tumor activity of pinecone extract resides in its ability to alter immune function in favor of a Th1 response, and in its ability to activate dendritic and macrophage cells. See Appendix A at p. 10, lines 3-6.

9. I understood at the time of filing the grant application of Appendix A (June 1, 1999) that the only known function of dendritic cells was to present antigen to T-cells, and that the mature dendritic cells found in lymphoid tissues were generally known to be by far the most potent stimulators of naïve T cells. See Janeway "The Immune System in Health and Disease" 5<sup>th</sup> Edn., Garland Publishing, at p.307, §8-6, and Appendix A at p.7, ¶¶3-5, et seq.

10. It was my belief and opinion at the time of filing the proposal of Appendix A on June 1, 1999, that an agent which boosted dendritic cell function would also boost antigen presentation to T-cells, which is precisely the function of a vaccine adjuvant. See Janeway at p.576 ("The potency of dendritic cells in activating T-cell responses provides the rationale provides the rationale for yet another strategy for

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vaccinating against tumors. The use of antigen-pulsed autologous dendritic cells to stimulate therapeutically useful T-cell responses to tumors has been developed in experimental models, and there have been initial trials in humans with cancers," and Appendix A at p.7, ¶¶3-5 et seq.

11. It was my belief and opinion at the time of filing the proposal of Appendix A that elucidation of the mechanism of action of PC extract would aid in the use of pine cone extract to complement current anti-cancer therapies.

12. We demonstrated in a pilot study prior to the time of filing the proposal of Appendix A that oral delivery of pine cone extract to aged mice reduced the incidence of spontaneous tumor formation, consistent with our hypothesized mechanism of action of pine cone extract. See Appendix A at p.9, ¶5.

13. We proposed experiments in the proposal of Appendix A to test our hypothesis that exposure of murine dendritic cells to pine cone extract in vitro will prime the cells for anti-tumor activity in vivo. See Appendix A at p.5.

14. Thus, I declare that as of the date of submission of the proposal (June 1, 1999) we had conceived and were in possession of the invention as disclosed and claimed in the subject application for U.S. Patent, and were actively engaged in its reduction to practice.

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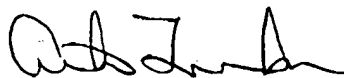
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15. That reduction to practice of the invention continued diligently from prior to the filing of the proposal of Appendix A to the filing of the above-captioned application for U.S. Patent, as shown by the statement of financial support provided by Ms. Diane Tippins, Business Manager of the Tampa Bay Research Institute, a copy of which is appended as Appendix C.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Date:

7/24/03



Akiko TANAKA, Ph.D.



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APPENDIX A



Tampa Bay Research Institute

A NOT-FOR-PROFIT ORGANIZATION DEDICATED TO CANCER & BIOMEDICAL RESEARCH

**TBRI BOARD OF  
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June 1, 1999

Commander

U.S. Army Medical Research and Materiel Command

ATTN: MCMR-PLF (BCRP99-Announcement)

1076 Patchel Street (Building 1076)

Fort Detrick, MD 21702

Dear Commander:

Enclosed please find the original and thirty (30) copies of the proposal entitled, "Dissecting the Legendary Anti-Tumor Activity of a Pine Complex: Activating the Anti-Tumor Potential of Macrophage and Dendritic Cells."

We have used our latest non-profit indirect cost rate of 95% for this proposal. However, a new indirect cost rate will have to be established upon receiving an award.

We thank you for your consideration of our request.

Sincerely,

Diane C. Tippins  
Business Administrator

**Enclosures**

Original and thirty copies of Proposal

Original and three copies of Cover Booklet

Five copies each of Technical and Public abstracts

Abstract computer disk

Nonprofit Rate agreement

P.I.

cc: William G. Bradley, Ph.D.

**Proposal Title:** Dissecting the Legendary Anti-Tumor Activity of a Pine Complex: Activating the Anti-Tumor Potential of Macrophage and Dendritic Cells

**Principle Investigator:** William Guy Bradley, Ph.D.

For thousands of years humans have used the plants around them to help combat disease. The protective or healing properties of some of these plants has been associated with their ability to modulate immune function. It is therefore quite possible that many of the plants whose use has been propagated by folklore combat disease by modulating immune activity. By bolstering the immune system these products could also profoundly affect the development of human cancers. The extract from the cones of the pine, *Pinus parviflora* Sieb et. Zucc, appears to be such a product. In fact, because of its legendary anti-tumor potential, oral intake of pine cone extract is still a popular practice on the Kyushu Island of Japan. Based upon published reports and observations made recently in our laboratory, we hypothesize that the host-mediated anti-tumor activity of the pinecone extract (PC, Pine Complex) resides in its ability to alter immune function in favor of a Th1 response and to activate dendritic cells (DC) and macrophage.

To date many studies, including several of our own, have described the activity of the pinecone extract. Studies have shown that the PC is capable of inducing activation of murine and human macrophage/dendritic cells. When PBMC from a person who had taken PC or 2 weeks were stimulated with concanavalin A in vitro we observed a significant increase in cell adherence and spreading. Administration of the extract to lactating SHN mice prevented an increase of mouse mammary tumor virus (MMTV) in the milk of nursing mothers and reduced formation of MMTV-associated breast cancer. When the extract was delivered to another group of mice intraperitoneally, it was found to effectively inhibit the growth of ascites sarcoma-180 cell tumors. When the extract was incubated with the sarcoma cells in vitro, there was no detectable effect on the growth or viability of the tumor cells. From these results it was concluded that the anti-tumor activity observed in vivo reflects activation of host-mediated anti-tumor activity. Based on these results we recently investigated the ability of PC to alter immune function in aged mice. We found that treatment, by supplying PC continuously in their drinking water for 30 days, of the mice lead to alteration of splenocyte cytokine production. Specifically, PC enhanced expression of IL-12p70 by 94% and suppressed expression of IL-10 by 88%. Our findings suggest that the legendary anti-tumor might be rooted in its ability to favor a protective Th1 response, a response that would be associated with activation of macrophage and dendritic cells and would increase their ability to process and present tumor antigens.

Central to this research proposal is experiments designed to investigate the ability of PC to modulate immune function and effect the development of cancer. For these reasons we will pursue the following two aims: (1) To determine the concentrations of PC that effectively inhibit tumor development in a well established murine tumor model and (2) To determine if exposure of murine dendritic cells to PC in vitro will prime them for anti-tumor activity in vivo. Since previous investigations have demonstrated that the mere presence of macrophage and DC infiltrating into tumors correlates with a better prognosis, the ability to stimulate the production of IL-12 and to activate DC and macrophage in vivo could prove to be extremely useful in the treatment of breast cancer. To identify and characterize the cells associated with PC-induced tumor regression, we will utilize histochemical, immunohistochemical, and FACS analysis. To enhance the examination of tumor development and visualization of tumor cells and tumor margins we will use tumor cell lines that have been engineered to express high levels of green fluorescent protein (GFP).

Since the use of PC to combat cancer in humans has been predominately based upon anecdotal evidence there exists a need for well-controlled scientific experiments. By performing a thorough investigation of PC's affects on the immune system and its anti-cancer activity, it is quite possible that we may substantiate the anecdotal reports and thereby move the use of PC from the realm of folklore into the complement of therapies used to combat diseases like breast cancer.



- **Public Abstract**

**Proposal Title:** Dissecting the Legendary Anti-Tumor Activity of a Pine Complex: Activating the Anti-Tumor Potential of Macrophage and Dendritic Cells

**Principle Investigator:** William Guy Bradley, Ph.D.

For thousands of years humans have used the plants around them to help combat disease. The protective or healing properties of some of these plants has recently been associated with their ability to modulate immune function. It is therefore quite possible that many of the plants whose use has been propagated by folklore combat disease by modulating the immune system. By bolstering the immune system these products could also profoundly affect the development of human cancers. An extract from pinecones appears to be such a product. In fact, because of its legendary anti-tumor potential, the drinking of a tea made from the pinecone is still a popular practice on the Kyushu Island of Japan.

Based upon published reports and observations made recently in our laboratory, we hypothesize that the anti-cancer activity of an extract from pine cones (PC, Pine Complex) resides in its ability to alter immune function and to specifically stimulate the activation of cells capable of recognizing and destroying cancer cells. Previous research has demonstrated that within well-controlled experiments the pinecone extract is capable of combating a number of diseases, including breast cancer. Recent research performed in our laboratory has found that the extract is able to significantly stimulate the immune system of aged mice (we chose aged mice because the incidence of cancer and other diseases in humans increases greatly as we age) to secrete factors that would be beneficial in the establishment of an anti-cancer immune response.

Central to this research proposal is experiments designed to investigate the ability of PC to stimulate immune function and suppress or prevent the development of cancer. For these reasons we will pursue the following two aims: (1) To determine the concentration of PC that effectively inhibits cancer production in a well established mouse model and (2) To determine if we can activate the anti-cancer potential of immune cells with PC in the test tube and then by injecting them directly into a tumor induce the regression of the cancer.

Since the use of PC to combat cancer in humans has been predominately based upon anecdotal evidence there exists a need for well-controlled scientific experiments. By performing a thorough investigation of PC's affects on the immune system and its anti-cancer activity, it is quite possible that we may substantiate the anecdotal reports and thereby move the use of PC from the realm of folklore into the complement of therapies used to combat diseases like breast cancer.

## Statement of Work

**Bradley, William G.**

### **Dissecting the Legendary Anti-Tumor Activity of a Pine Complex: Activating the Anti-Tumor Potential of Macrophage and Dendritic Cells**

**Task 1:** To determine the concentrations of PC that effectively inhibit tumor development in BALB/c murine tumor model (months 1-24)

- Create the C51 tumor cell line that constitutively express the green fluorescent protein at high levels (months 1-7)
- Establish minimal number of CT-26 and C51 cells required to induce tumors in 100% of the mice within 2 weeks (months 7-9)
- Begin testing different concentrations of PC for its ability to inhibit or reduce tumor formation (months 9-15)
- Examine tumor architecture by using histological and immunohistological techniques, and FACS analysis (months 9-21)
- Statistical analysis of results (months 21-24)

**Task 2:** To determine if exposure of murine dendritic cells to PC in vitro will prime them for anti-tumor activity in vivo (months 24-60).

- Isolation and characterization of dendritic cells (months 24-30)
- Determination of appropriate concentration of PC to activate the isolated dendritic cells in vitro (months 24-30)
- Establish appropriate concentration of PC that inhibits tumor formation in <50% of the mice (months 30-33)
- Begin testing for the most effective timing of injection of the in vitro activated dendritic cells (months 33-45)
- Examine tumor architecture by using histological and immunohistological techniques, and FACS analysis (months 45-57)
- Statistical analysis of results (months 57-60)

### **Proposal Relevance and Impact Statement**

An extract from the cones of the pine, *Pinus parviflora* Sieb et. Zucc, has been used over the centuries by people in China, Germany, Greece, and Japan to combat diseases. Its recent rediscovery was based upon its association with the significant reduction in the incidence of cancer in people that used it as a component of their traditional medicine. Since the use of the extract to combat cancer in humans has been predominately based upon anecdotal evidence there exists a need for well-controlled scientific experiments. By performing a thorough investigation of PC's affects on the immune system and its anti-cancer activity, it is quite possible that we may substantiate the anecdotal reports and thereby move the use of PC from the realm of folklore into the complement of therapies used to combat diseases like breast cancer.

## **Proposal Body**

### **I. Background**

Breast cancer is the second leading cause of deaths in women. It is estimated that 1 in 11 women will eventually develop this disease. While science has made significant progress in the early detection and treatment of breast cancer, many of the currently approved treatments negatively impact the immune system (1-5). Unfortunately, impaired immune function is associated with poor prognosis in breast cancer (6-8). It would therefore seem logical that bolstering the immune system and specifically inducing an anti-cancer immune response would improve the outcome of cancer therapy. This is especially true in the aged population, where there is recognized a strong correlation between the increase incidence of cancers and a senescing immune system.

#### **Immunosenescence**

It has been well established that as humans and mice age, overall immune function declines. Concomitant with this decline is an increase in the incidence of cancer and other diseases. It has therefore been speculated that prevention or reversal of this decline in immune function could possibly reduce the incidence of cancers and other age-related diseases (9-12). One of the immunologic changes associated with aging is T cell dysfunction. This T cell dysfunction is recognized as the reduced ability of aged T cells to proliferate in response to mitogens and antigens and as an imbalance in the T helper (Th) patterns of cytokine expression (13). This aging-associated T cell dysfunction is associated with thymus involution and an increase in the CD4+ T cell population having a memory phenotype (14). When the peripheral blood mononuclear cells (PBMC) from young and adult humans and PBMC and splenocytes from young and adult mice are stimulated in vitro, they produce a T helper type 1 (Th1) pattern of cytokine expression. However, when the PBMCs from aged humans and PBMCs and splenocytes from aged mice are stimulated in vitro, they produce a T helper type 2 (Th2) pattern of cytokine expression (15-20). The Th1 pattern of cytokine expression is characterized by the production of interleukin 2 (IL-2), IL-12, and interferon gamma (IFN- $\gamma$ ) and is necessary for a proper cellular immune response and the ability to combat cancers, while the Th2 pattern of cytokine expression is characterized by the production of IL-4, IL-5, IL-6, and IL-10 and is necessary for a proper humoral immune response.

#### **Interleukin 12, Dendritic Cells and Macrophage**

The expression of IL-12 is associated with the Th1 type of immune response. Interleukin 12 has been found to affect T cell and natural killer cell functions, including induction of IFN- $\gamma$  production and the enhancement of cell-mediated cytotoxicity, and to play a major role in the induction of the Th1 response and suppression of the Th2 response (21-23). Since IL-12 can help activate a Th1 response, it has been speculated that it could be used to treat diseases like cancer (24). Several studies in fact have demonstrated that systemic administration of IL-12 has anti-tumor effects in animal models (25-27).

It is currently believed that IL-12 induces an anti-tumor state by activating professional antigen-presenting cells (APC, macrophage and dendritic cells). In fact, it has been demonstrated that tumor antigens are presented to T cells not by the tumor cells themselves but by host APC (28). During antigen-induced immune responses, dendritic cells (DC) take up antigen, migrate through the lymphatic system or blood stream to the lymphoid organs, and present the antigen to T cells. It appears that the capacity to internalize and process antigen is a constitutive property of DC present in non-lymphoid organs (29).

In the absence of IL-12, antigen-presenting cells infiltrating a tumor can capture tumor antigens but appear to present the antigens as new epitopes arising from normal self-antigens rather than from a threatening tumor (30). In the presence of IL-12, antigenic determinants from the tumors are recovered by professional antigen presenting cells (APC) and then processed and delivered to cytotoxic T cells. It appears that DC and macrophage are the only cells that can do this in response to IL-12 (31,32). This process of antigen processing and

presentation is so critical to establishing an anti-tumor response that the mere presence of macrophage and DC infiltrating tumors has been correlated with a better prognosis (33). Therefore, the ability to stimulate the production of IL-12 and to activate DC and macrophage could prove to be extremely useful in the treatment of breast cancer. In several studies it has been demonstrated that activation of DC in vitro, in the presence of tumor antigen and specific cytokines, can induce tumor-specific cytotoxicity when used as a tumor vaccine (34-37). But how do we accomplish this in vivo? Based upon years of accumulating anecdotal evidence and preliminary studies in our laboratory, we believe that an extract from the cones of pine may be able to inhibit the production of IL-10, stimulate the production of IL-12, and activate DC and macrophage in vivo, thereby inducing anti-tumor activity.

### Origin of the Pinecone Extract

For thousands of years humans have used the plants around them to help combat disease (38). The protective or healing properties of some of these plants has recently been associated with their ability to modulate immune function. It is therefore quite possible that many of the plants or plant products whose use has been propagated by folklore, combat disease by modulating the immune system. By bolstering the immune system these products could also profoundly affect the development of human cancers. The extract from the cones of the pine, *P. parviflora* Sieb et. Zucc, which contains perylpropanoid polymers complexed with polysaccharides, appears to be such a product. In fact, because of its legendary anti-tumor potential, oral intake of the *Pinus parviflora* Sieb et. Zucc extract is still a popular practice on the Kyushu Island of Japan (39).

### In vitro Activity of the *Pinus parviflora* Sieb et. Zucc

Extract To date several studies, including several of our own, have described the in vitro activity of the *Pinus parviflora* Sieb et. Zucc extract. Studies have shown that an alkaline extract from *Pinus parviflora* Sieb et. Zucc is capable of inducing the activation of a mouse macrophage cell line (40). The extract was found to induce morphological spreading and stimulated the NBT-reducing activity of peritoneal macrophage. When human peripheral blood monocytes were primed with the extract and then stimulated with LPS, a significant increase in TNF mRNA expression was observed (41). Recently, when we stimulated human PBMC in vitro with increasing concentrations of either concanavalin A or a commercially available pinecone extract, (PC; Pinextra, Vitamune, Tampa, Florida), we detected a very highly significant increase in the attachment and spreading of dendritic/macrophage in the samples treated with PC (manuscript in preparation).

The extract from *Pinus parviflora* Sieb et Zucc has also been shown to inhibit virion-associated RNA dependent RNA polymerase, inhibit both forward- and reverse-transcription of HIV in vitro, inhibit HIV replication in T cell and monocytic cell lines in vitro (42), and inhibit plaque-formation in cells infected with either influenza or herpes simplex virus (43,44). While these in vitro activities are quite interesting, we are presently focusing our activities toward understanding how the extract works in vivo.

### In vivo Activity of the *Pinus parviflora* Sieb et. Zucc Extract

To date, only a few studies describing the in vivo activity of the pinecone extract have been reported. These studies have reported the antimicrobial, antiparasitic, antiviral, and antitumor activity of an alkaline extract from the *Pinus parviflora* Sieb et. Zucc. Oh-Hara et al. demonstrated that pretreatment of mice with Fraction VI (Fr. VI) of the *Pinus parviflora* Sieb et. Zucc extract was capable of inducing a potent antimicrobial response against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Candida albicans* (45,46). Abe et al. reported that similar fractions of the pinecone extract were capable of protecting both young and infant mice from parasitic (*Hymenolepis nana*) infection (47).

### Analysis of Toxicity

In 1987, Sakagami et al. described the preparation of several biologically active fractions from *Pinus parviflora* Sieb et Zucc (48). When acute and subacute toxicity tests were performed on Fr VI, it was discovered to be

moderately toxic when delivered intravenously to mice. The LD<sub>50</sub> value of the extract on these mice was approximately 60 mg/kg. However, when Fr VI was delivered orally to mice, no toxic effects were noted at doses as high as 405 mg/kg (41). When doses ranging from 5 mg/kg to 405 mg/kg was administered daily for 30 days there was observed no alteration of the hepatic P-450 system (41).

#### **Anti-tumor activity of the *Pinus parviflora* Sieb et Zucc Extract**

The administration of Fr. VI of the extract, either intravenously or orally to lactating SHN mice (a strain of mice that produces mouse mammary tumor virus in their milk and develops MMTV-associated breast cancer), appeared to prevent an increase of mouse mammary tumor virus (MMTV) in the milk of nursing mothers (49). When mice that had spontaneously developed mammary tumors were treated by intravenous injection of Fr. VI, it was found that the treatment effectively reduced the activity of thymidylate synthetase and thymidine kinase in cells derived from the tumors (50).

When the *Pinus parviflora* Sieb et Zucc extract was delivered intraperitoneally at a concentration of 20 mg/kg it was found to effectively inhibit the growth of ascites sarcoma-180 cell tumors in mice and increased the mean survival time from 16-18 days (control group) to 31-51 days (treated group) (48). When the *Pinus parviflora* Sieb et Zucc extract (at concentrations up to 500 ug/ml) were incubated with the sarcoma-180 cells in vitro, there was no detectable effect on the growth or viability of the tumor cells. From these results it was concluded that since the extract had no apparent effect on the tumor cells in vitro, the anti-tumor activity observed in vivo reflects activation of host-mediated anti-tumor activity (48).

Because the report by Sakagami et al. suggested that the extract's anti-tumor activity is likely to be associated with the ability to modulate immune function, we recently investigated the potential of oral administration of PC to alter immune function. Since we realized that enhancement of immune function and the induction of anti-tumor activity in the aged would be especially important, we investigated the ability of oral administration of PC to aged mice to alter the ability of their splenocytes to produce a number of cytokines (manuscript in preparation). We found that oral administration of PC to aged B6C3F1/Nnia mice profoundly altered cytokine production by their concanavalin A-stimulated splenocytes. Treatment, by supplying PC continuously in their drinking water for 30 days, of the aged mice lead to enhanced expression of IL-12p70 (94% above levels in non-treated mice) and suppression of IL-10 (88% of the levels produced by non-treated mice). Our findings that the oral administration of PC to aged mice suppressed the production of IL-10 and enhanced the production of IL-12, suggest that the legendary anti-tumor potential ascribed to the pine cone extract might be rooted in its ability to counteract the Th2 pattern of cytokine expression and favor a protective Th1 response. Such a response, if it occurs in vivo, would likely favor activation of macrophage and dendritic cells and increase their ability to process and present tumor antigens.

In a small pilot study (only 5 mice per treatment group) currently in progress, the oral delivery of PC to aged B6C3F1/Nnia mice (23-25 months old) appears to have enhanced their longevity and overall health. While several mice in the group receiving only water have developed spontaneous tumors and expired, none of the mice receiving PC have developed spontaneous tumors nor have any expired. The mice are currently 30-32 months old (expected life span for B6C3F1/Nnia is reported to be 35-36 months). While these findings are very interesting, this study utilized a minimum of mice and was designed to look only at longevity and not specific alterations of immune function or the establishment of an anti-cancer response.

While the anti-tumor activity of PC in humans is legendary on the Kyushu Island of Japan, and while the health benefits of PC that have been described in the Greek Herbal of Dioscorides (published in the first century A.D.) are compelling, much of the evidence remains anecdotal. By performing a thorough investigation of PC's effects on the immune system and its anti-cancer activity, it is quite possible that we may end up substantiating the anecdotal reports and thereby move the use of PC from the realm of folklore into the complement of therapies

used to combat cancer.

## II. Hypothesis/Rationale/Purpose

Based upon published reports and observations recently made in our laboratory, we hypothesize that the host mediated anti-tumor activity of PC resides in its ability to activate dendritic cells and macrophage. Since the rediscovery of the pinecone extract was due to its legendary anti-tumor activity we anticipate that by elucidating its mechanism of action the use of PC to complement current anti-cancer therapies is forthcoming.

## III. Objectives (specific aims)

Central to this research proposal is experiments designed to investigate the ability of PC to modulate immune function and effect the development of cancer. For these reasons we wish to pursue the following aims.

**Aim 1. To determine the concentrations of PC that effectively inhibits tumor development in the BALB/c murine tumor model.** The rediscovery of the *Pinus parviflora* Sieb et. Zucc extract was due to its remarkable anti-cancer activity in humans. In order to dissect how PC effects tumor development, we will determine the concentrations of PC that effectively suppress tumor development in the widely accepted murine tumor model (BALB/c CT26 adenocarcinoma). We will utilize histochemical, immunohistochemical, and FACS analysis to characterize and identify the cells involved in tumor suppression/regression. To enhance the histological examination of tumor development we will use tumor cell lines that have been engineered to express high levels of green fluorescent protein (GFP). The constitutive expression of GFP in the tumor cells will allow direct visualization of tumor cells and tumor margins under fluorescent microscopy.

**Aim 2. To determine if exposure of murine dendritic cells to PC in vitro will prime them for anti-tumor activity in vivo.** Since it has been established that in vitro activation of tumor antigen-pulsed DC are capable of inducing tumor-specific cytotoxicity and providing protection against subsequent inoculation of tumor cells in vivo, we will determine if exposure of dendritic cells to PC in vitro, followed by continuous oral administration of PC, will prime them for establishing an anti-tumor state in vivo. We will utilize histochemical, immunohistochemical, and FACS analysis to characterize and identify the cells involved in tumor suppression/regression. To enhance the histological examination of tumor development we will use tumor cell lines that have been engineered to express high levels of green fluorescent protein (GFP). The constitutive expression of GFP in the tumor cells will allow direct visualization of tumor cells and tumor margins under fluorescent microscopy.

## IV. Methods

**Aim 1. To determine the concentrations of PC that effectively inhibits tumor development in BALB/c murine tumor model.**

**Rationale:** Since the remarkable anti-cancer activity of PC has been predominately based upon anecdotal evidence there exists a need for well controlled scientific experiments to substantiate its legendary anti-cancer activity. In order to mimic the development of cancer in humans in a more controlled environment, we will use the well-characterized BALB/c CT26 and C51 (adenocarcinomas) tumor model to investigate the concentrations of PC that effectively delays, suppresses, or induces regression of tumor development. The results obtained from this series of experiments will allow us to better formulate the complementary use of PC in the treatment of cancers.

**Experimental Design:** BALB/c mice will be obtained from Jackson Laboratories. Mice will be housed in

AAALAC approved facilities associated with the University of South Florida (Tampa, Florida). This research protocol has been approved by the USF's IACUC and has been assigned the file # 1442. Mice will be grouped and treated with PC at the concentrations shown in Table I by supplying it continuously in their drinking water. Tumor formation will be initiated by injecting  $10^4$  CT-26 or C51 cells subcutaneous in their flank. This dose of tumor cells reproducibly induces detectable tumor formation in 80-100% of mice within 10 days of injection. Treatment with PC will begin either 2 weeks prior to, at the same time as, or 1 week after injection with  $10^4$  tumor cells. The treatment schedule will continue until tumors in the control group (0 ug/ml) expand to >10mm in diameter, after which the tumors will be removed and examined histologically using conventional and immunohistochemical stains. Specifically we will prepare frozen and paraffin sections from tissues in the vicinity of the tumor cell injection and/or resulting tumors and examine them by immunohistochemistry for infiltrating T-helper cells (CD4+/L3T4+), cytotoxic T cells (CD8+/Lyt-2+), NK cells (asialo-GMI+), B cells (CD45R+/B220+), or dendritic/macrophage/monocytes (CD86+/B7-2+). We will also look for the presence of newly activated T cells and NK cells displaying the very early activation antigen, CD69. The frozen and paraffin sections and the hematoxylin and eosin staining of paraffin sections will be performed by staff of All Children's Hospital's Department of Pathology (St. Petersburg, FL). For histologic analysis, tissues will be fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 2-5um, and stained with hematoxylin-eosin. For immunocytochemistry, tissues will be embedded in OCT compound, snap-frozen in liquid nitrogen, and preserved at -80°C until sectioned. Cryostat sections of 5 um will be fixed in acetone and immunostained with purified rat mAb to: CD4/L3T4, CD8/Lyt-2, asialo-GMI, CD45R/B220, CD86/B7-2 or CD69 (Southern Biotechnology Associates, Inc). Each of these antigen specific mAbs will be conjugated with phycoerythrin (R-PE). To develop the tissue sections, reagents available in the Vectastain ABC kits (Vector Laboratories) will be used. The tissue sections will be counterstained with hematoxylin and then examined by light and fluorescence microscopy using a Nikon Eclipse E600 microscope equipped with a Microflex U-III camera system.

To enhance the histological examination of tumor development we will use CT26 and C51 tumor cells that have been engineered to express high levels of green fluorescent protein, thereby allowing for direct visualization of tumor cells under fluorescent microscopy. Using fluorescent microscopy we will be able to detect the tumor and its margins and we might also be able to detect small numbers of tumor cells at the site of injection in the mice that appear to have suppressed tumor formation. When we superimpose the results from light microscopy with the fluorescent microscopy it will be easy to determine the specific locations of tumor infiltrating cells.

To determine how PC may have influenced tumor production we will also analyze cytokine expression by the splenocytes isolated from each mouse that either: (1) suppressed tumor formation, (2) induced tumor stasis, or (3) induced tumor regression. We will compare the levels of cytokine production in these 3 groups of mice to the levels detected in the control mice with tumors.

To examine the effects of oral administration of PC on cytokine expression we will analyze the ability of splenocytes and lymph node cells to respond to anti-CD3 mAb, concanavalin A (ConA), or lipopolysaccharide (LPS) mitogen in vitro. Mice will be sacrificed, spleens and lymph nodes will be removed aseptically, and single cell suspensions will be prepared individually for each mouse using a Stomacher 80 Laboratory Blender (Seward Medical, London, England). Cell suspensions will be passed through a fine mesh filter prior to use. The cells will be enumerated using a Coulter Counter and viability will be assessed by examination of trypan blue dye exclusion using a hemocytometer.

Splenocytes and lymph node cells will be diluted to  $2 \times 10^6$  per ml in serum free media (media is RPMI 1640 containing, 1x Nutridoma SR (Boehringer Mannheim), 10 mM Hepes, 1% L-glutamine, 1% penicillin/streptomycin, and 50 uM 2-mercaptoethanol) containing either 10ug/ml anti-CD3 mAb (clone 145-2CII), 5ug/ml ConA or 20ug/ml LPS and then incubated at 37C in an atmosphere containing 5% CO<sub>2</sub>.



Triplicate cell cultures will be prepared from each cell source. After 48 hr the supernatants will be harvested and stored at -80°C. The amount of IL-1, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12p70, TNF- $\alpha$ , and IFN- $\gamma$  in the supernatants will be measured by ELISA using commercially available kits. The assays will be performed according to the manufacturer's instructions.

All in vitro cytokine expression experiments will be performed independently in triplicate. The significance of differences between the levels of cytokine expression in each treatment group will be statistically evaluated using a two-tailed Student's t-test. The tumor takes will be analyzed by Fisher's exact method and the latency/survival time will be analyzed using the two-tailed Wilcoxon's signed rank test.

**Expectations and Feasibility:** The use of CT26 and C51 tumor cell lines in BALB/c as tumor models have been well established and are extremely reproducible. The CT26 and C51 are colon adenocarcinoma cell lines derived from BALB/c mice treated with N-nitroso-N-methylurethane and 1,2-dimethylhydrazine, respectively (51). We choose to use these tumor cell lines because they were both derived from BALB/c mice and they share transplantation and CTL-recognition antigens (52). However, CT26 is significantly more resistant than C51 to systemic administration of IL-12 (53), an experimental treatment that has been demonstrated to improve the survival of mice bearing a variety of tumors (54). The greater resistance of CT26 to cytotoxic attack is likely to be associated with the ability of CT26 to induce IL-10 production by the infiltrating T cells when IL-4 is present (32). Since our previous investigations demonstrate that the major effect of PC is to induce IL-1 2 production and suppress IL-10 production in vitro in stimulated splenocytes, it is likely that PC will also stimulate the production of IL-12 and suppress the production of IL-10 in vivo and lead to tumor regression. Having many years of experience working with such tumor models we do not anticipate any technical difficulties. The CT26/GFP cell line will be provided by Dr. Robert Siliciano (John's Hopkins University). According to the observations in Dr. Siliciano's laboratory, the presence of GFP in the CT26 cells does not appear to significantly affect their ability to develop tumors in vivo or the immune response against such tumors.

The results obtained from this series of experiments may provide scientific evidence that supports the historical claims that PC has or induces anti-tumor activity or at least will provide a foundation from which we can begin investigating PC's specific mechanism of action.

**Aim 2. To determine if exposure of murine dendritic cells to PC in vitro will prime them for anti-tumor activity in vivo.**

**Rationale:** Previous investigations have demonstrated that the mere presence of macrophage and DC infiltrating into tumors correlates with a better prognosis (33). Therefore, the ability to stimulate the production of IL-1 2 and to activate DC and macrophage in vivo could prove to be extremely useful in the treatment of cancer. In several studies it has been demonstrated that activation of DC in vitro, in the presence of tumor antigen and specific cytokines, can induce tumor-specific cytotoxicity when used as a tumor vaccine (34-37). In Aim I we will determine if PC's anti-tumor activity is associated with its ability to induce macrophage and DC infiltration into tumors. Even if we find that oral administration of PC is capable of 100% tumor suppression/regression we are aware that it may not work for all types of cancer and that an alternative protocol should be investigated.

In Aim 2 we would like to determine the feasibility of combining the oral administration of PC (Aim 1) with in vitro dendritic cell activation in an attempt to maximize the stimulation of anti-tumor activity. However, in contrast to previous investigations where the DC were "loaded" in vitro with mock tumor antigens, we wish to determine if dendritic cells activated in vitro by PC and then injected directly into the tumor or at a distant site, can recognize the tumor, process the tumor antigens, and establish anti-tumor activity. This scenario may be more closely representative of the human condition and therefore needs to be investigated.

## Experimental Design

For these experiments we will utilize 2 groups of BALB/c mice (8 weeks old). One group will be the source of splenic dendritic cells and the other will be the tumor test group.

**Activation of DC in vitro.** Mice will be sacrificed, spleens will be removed aseptically, and single cell suspensions will be prepared using a Stomacher 80 Laboratory Blender (Seward Medical, London, England). Cell suspensions will be passed through a fine mesh filter prior to use. Low-density cells will be isolated by centrifugation over a 60% Percoll gradient (density = 1.076). Dendritic cells will be further enriched by differential adherence by incubating the cells on 100mm tissue culture plates in medium containing 5% fetal calf serum (FCS) for 2 hours at 37°C. The nonadherent cells will be removed and the remaining cells will be cultured overnight in medium containing 5% FCS. Twenty-four hours later, floating cells (DC) will be collected. The cells will be transferred to media containing 5% FCS and PC at 50ug/ml. The cells will be cultured for 24-48 hours. The cells will be harvested by scraping them from the tissue culture dish and then washed with 0.9% saline (normal saline). The cells will be suspended in normal saline at a concentration of  $1 \times 10^6$  cells/ml.

**Oral administration of PC.** The dose of PC that was found in Aim 1 to inhibit tumor formation by <50% when delivery was initiated at the same time as tumor cell injection will be used.

**Tumor Induction/Treatment.** On Day 0 fifty mice will be injected with  $10^4$  CT26/GFP cells subcutaneous in the flank and then separated into 5 treatment groups (Figure 1). On Day 0, Group 1 will receive an intratumor injection of  $10^5$  PC-treated dendritic cells and PC in their drinking water. Two days post tumor cell injection, Group 2 will receive an intratumor injection of  $10^5$  PC-treated dendritic cells and PC in their drinking water. Four days post tumor cell injection, Group 3 will receive an intratumor injection of  $10^5$  PC-treated dendritic cells and PC in their drinking water. Six days post tumor cell injection, Group 4 will receive an intratumor injection of  $10^5$  PC-treated dendritic cells and PC in their drinking water. Eight days post tumor cell injection, Group 5 will receive an intratumor injection of  $10^5$  PC-treated dendritic cells and PC in their drinking water. The mice will be analyzed on a daily basis for tumor growth. The size of the tumors will be determined with small calipers by measuring the diameter. The experiment will continue until the tumors are 20 mm in diameter or if no tumors have developed after 60 days.

**Statistical analysis.** The tumor takes will be analyzed by Fisher's exact method and the latency/survival time will be analyzed using the two-tailed Wilcoxon's signed rank test.

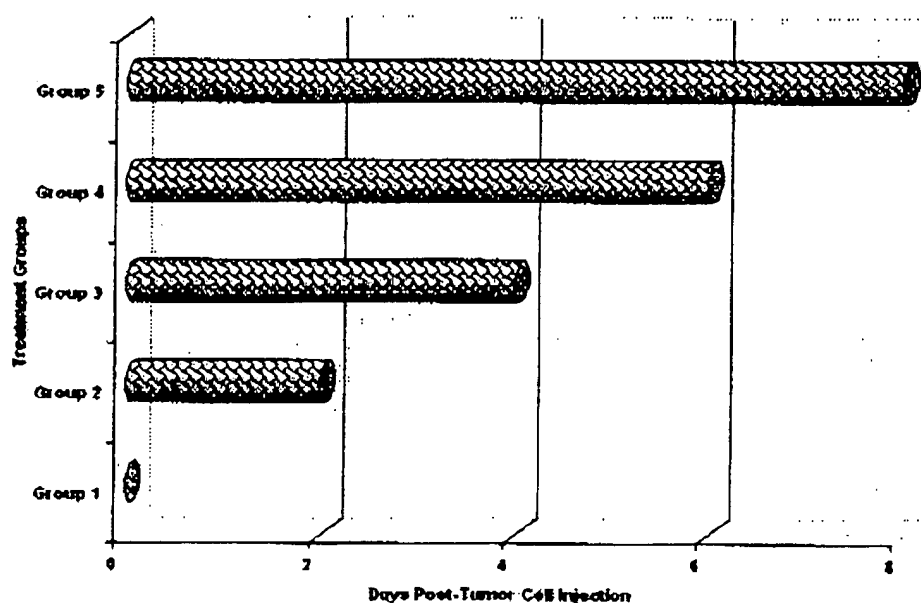
**Expectations and Feasibility:** The experiments described in Aim 2 will allow us to determine if the combination of orally administered PC with the intratumor injection of in vitro PC-stimulated dendritic cells is effective at inducing anti-tumor activity. The experimental design will allow us to test a variety of scenarios. By injecting the DC and beginning oral administration of PC 0 and 2 days post tumor cell injection we will mimic a condition similar to that in patients with a minimal tumor burden (either because of early detection or resection of the majority of the tumor by surgery). By injecting the DC and beginning oral administration of PC 4, 6, and 8 days post tumor cell injection we will mimic a condition similar to that found in patients where significant tumor growth has occurred.

If we fail in Aim 1 to find an effective concentration of PC that induces tumor suppression or regression we will then determine if the combination treatment (Aim 2) using PC is effective at inducing anti-tumor activity. However, since the rediscovery of the pinecone extract was due to its legendary anti-tumor activity we anticipate that positive results will be obtained from Aim 1. The results obtained from both Aim 1 and Aim 2 will provide us information that will help in deciphering the mechanisms by which PC induces anti-tumor activity.

## V. Figures and Tables

**Table 1. Treatment schedule for each group of mice injected with  $10^4$  tumor cells**

Timing of PC Treatment	Number of mice to be utilized in each group				
	Group 1	Group 2	Group 3	Group 4	Group 5
2 weeks prior to tumor cell injection	10	10	10	10	10
Same time as tumor cell injection	10	10	10	10	10
1 week post tumor cell injection	10	10	10	10	10



**Figure 1.** Mice in each treatment group will be injected with tumor cells on Day 0 and then 2, 4, 6, or 8 days later they will receive an injection of PC-activated dendritic cells and will start receiving PC continuously in their drinking water.

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U.S. Patent Application No.: 09/964,240  
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APPENDIX B

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the Anti-Tumor Potential of Macrophage and Dendritic Cells**

Please cite your proposal identification number in any further correspondence.  
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Thank you for your interest.

Kenneth A. Bertram, M.D., Ph.D., F.A.C.P.  
Lieutenant Colonel, U.S. Army Medical Corps  
Director, Congressionally Directed Medical  
Research Programs



U.S. Patent Application No.: 09/964,240  
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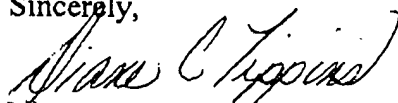
Akiko Tanaka, Ph.D.  
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Christopher J. Kay, Ph.D.  
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Dear Dr. Kay:

This letter is to verify that the project related to Pine Cone Extract was funded during the period of January 10, 2001 through September 26, 2001 through Tampa Bay Research Institute (TBRI) Program Funds.  
A foundation grant as well as TBRI Program funding covered funding prior to this time period.

Sincerely,



Diane C. Tippins  
Business Manager